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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01K 67/033, A23K 1/18

A1

(11) International Publication Number: WO 95/18527

(43) International Publication Date: 13 July 1995 (13.07.95)

(21) International Application Number: PCT/GB95/00021

(22) International Filing Date: 6 January 1995 (06.01.95)

(30) Priority Data:
9400271.4 7 January 1994 (07.01.94) GB

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published
With international search report.

(54) Title: NOVEL FEEDS FOR USE IN AQUACULTURE

(57) Abstract

The invention relates to nematodes grown on a liquid culture medium containing a fish oil, and to the use thereof in an aquaculture feed composition for marine organisms.

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NOVEL FEEDS FOR USE IN AQUACULTURE

This invention relates to marine aquaculture and more particularly to feeds suitable for the growth and development of marine fish and crustacea such as *Penaeus* species (penaeids) for example *Penaeus indicus* and *P. monodon.*

P. indicus passes through six nauplii, three zoeal (Z) and three mysis (M) stages until it metamorphoses into post-larvae (PL). The nauplius stage is non-feeding but at the zoeal stages penaeid prawns are initially fed directly on algae followed by either rotifers and/or *Artemia* during the mysis stages. Subsequently, the post-larvae are weaned onto synthetic diets. Some fish species are fed on a similar sequence of diets. The provision of suitable phyto- and zooplankton feed for the mass culture of the larval stages of fish and prawn species accounts for a highly significant proportion of the production costs of commercial hatcheries. Not only is the culture of these organisms extremely expensive, their nutritional quality is subject to considerable variation. This latter problem in conjunction with difficulties of co-ordinating sufficient supplies of algae and rotifers at the appropriate time can lead to massive losses of cultured species during the larval and postlarval stages.

Artemia cysts are harvested naturally from hypersaline lakes but problems of nutritional quality and contamination by pesticides and heavy metals are common. Supplies and costs of cysts tend to fluctuate and with the proposed increased development of commercial hatcheries in Europe, America and Asia there is increasing demand for suitable alternative larval feeds.

Nematodes, which are small free-living roundworms, have been suggested as a feed replacement for both rotifers and *Artemia.* Recently, there has been a demonstration of

nematodes as a possible replacement for *Artemia* in penaeid hatcheries (Biedenbach, J.M., Smith, L.L., Thomsen [redacted]. and Lawrence, A.L. Use of the nematode [redacted] *anagrellus redivivus* as an *Artemia* replacement in a larval penaeid diet. Journal of the World Aquaculture Society, 20, 61-71, 1989). Biedenbach et al found that a feed comprising algae and the nematode *P. redivivus* was a suitable substitute for a diet of *Artemia* and algae for rearing of penaeid larvae. The use of nematodes alone was not considered possible because they were known to be deficient in fatty acids such as docosahexenoic acid (22:6 ϖ3) and eicosapentaenoic acid (20:5 ϖ3) which are particularly beneficial to penaeid larvae. It was considered by Biedenbach et al that contributions from the algae may have corrected for possible fatty acid deficiencies in the nematodes. Biedenbach et al mass-produced nematodes on a solid medium which consisted of a paste of wheat flour and corn meal. However, this method is not suitable for mass production of nematodes on an industrial scale and they concluded that mass culturing of suitable quantities of nematodes is the major limiting factor for commercial applications.

Subsequently, it was found that the content of highly unsaturated fatty acids (HUFA), including docosahexenoic acid (22:6 ϖ3) and eicosapentaenoic acid (20:5 ϖ3), in *P. redivivus* could be increased by growing nematodes on a wheat flour paste to which a fish oil (cod liver) had been added (Rouse, D.B., Webster, C.D. and Radwin, I.A. Enhancement of the fatty acid composition of the nematode *Panagrellus redivivus* using three different media. Journal of the World Aquaculture Society 23, 89-95, 1992). However, incorporation of fish oil into the wheat flour paste reduced the yield of nematodes by approximately 50%. Thus the problem remains of mass-producing nematodes in suitable quantities for commercial applications in aquaculture. Rouse et al did not report any penaeid feeding trials using nematodes which had been produced on media containing fish oil.

WO 89/04602 (Biosys) describes a method for mass production in liquid culture of insect-killing

nematodes (Steinernematids and Heterorhabditids) based on a medium comprising an emulsifier, a source of vitamins [redacted] minerals, a source of triglycerides and a source of protein. Fish oil is mentioned as a possible source of triglycerides but no worked examples of suitable fish oils are presented nor is the possible effect of using fish oil on the HUFA content of nematodes discussed. Exemplification is limited to insect-killing nematodes and the use of the method for mass-production of free-living nematodes is not discussed.

It has now been found that nematodes which contain desirable quantities of HUFA can be produced by culture of nematodes on a liquid nutrient medium containing fish oil. The use of a liquid medium for culturing of nematodes allows them to be produced in large vessels such as fermenters thus enabling nematode mass production on a scale that is suitable for commercial applications. Examples of suitable fish oils include, but are not limited to, cod liver oil, herring oil, mackerel oil, sardine oil, Capelin oil, dog fish liver oil, salmon oil and pilchard oil.

The liquid nutrient medium will generally contain a source of protein, a source of triglycerides, a source of vitamins and minerals, and water. The source of protein will generally be present in a proportion of from 1 to 20% (w/w). Suitable examples are protein extracts, including animal- or plant-derived material such as dried animal organ extracts, fish meal, soy peptone and soy flour. Homogenised whole animal organs, such as kidney, may also be used. The source of triglycerides is a fish oil or a mixture of a fish oil and other oils or fats, e.g. vegetable oils such as corn, safflower, soy, sunflower, and rapeseed. The oil or fat component is generally present in a proportion of from 1 to 10% (w/w). The source of vitamins and minerals is, for example, yeast or yeast extract, and may be present in a proportion of from 0.1 to 5% (w/w). Other components which may be present in the medium include emulsifiers, such as lecithin, monoglycerides, polyoxyethylenesorbitan and fatty acid-carbohydrate esters, salts, antifoams, cholesterol or buffers, such as phosphate-buffered saline.

The invention is applicable to all species of nematode that are acceptable to marine organisms used in aquaculture. Examples of suitable nematode genera include but are not limited to, *Panagrellus, Turbatrix, Caenorhabditis, Monohystera, Theristis, Rhabditis, Phasmarhabditis, Heterorhabditis, Steinernema, Chromadora, Enoplus, Communis* and *Metoncholaimus.* Especially good results have been obtained with *Panagrellus redivivus,* a free living species of nematode found in soil and fermenting substrates.

Nematodes grown on media containing fish oil are herein described as enriched nematodes (EN). These are characterised by having an enhanced content of HUFA, especially docosahexenoic acid (22:6 ω3) and eicosapentaenoic acid (20:5 ω3) which are particularly beneficial to the larvae of penaeids and other species of fish and crustacea.

Carotenoid pigments can also play an important role in the nutrition of marine fish and crustacea in aquaculture. There is considerable evidence that carotenoids are involved in a variety of physiological functions in both fish and crustacea. In addition, a range of organisms, such as bream, carp, crustacea, goldfish, salmonids, tilapia and yellow tail, that are raised in aquaculture require carotenoids. The market value of penaeid post-larvae depends directly on their level of pigmentation. Conventionally, carotenoid pigments are included in the form of pellets or capsules in the feeds for post-larval stages of some crustacea and marine fish.

A further enhancement of the nutritional quality and attraction to the host organism of enriched nematodes has been obtained by incorporating suitable pigments in the nematode culture medium, especially astaxanthin. This leads to the production of nematodes which have incorporated the pigments into their guts. Such further improved nematodes are described herein as pigmented enriched (PEN). Examples of suitable pigments include, but are not limited to, carotenoids such as zeaxanthin and astaxanthin derived from fish and algae. Synthetic pigments, such as canthaxanthin, also are suitable. Such pigments may be present in the liquid

nutrient medium in a proportion of from 0.1 to 10% (w/w), preferably from 0.5 to 2.0% (w/w).

Surprisingly, EN nematodes have been found to enhance the growth of penaeid larvae. Furthermore, a dramatic and surprising improvement in the survival rate of *Penaeus indicus* larvae has also been observed employing EN and PEN nematodes with PEN nematodes of all diets tested giving the highest survival.

The present invention therefore comprises the mass production and use of nematodes of suitable nutritional composition as aquaculture feeds for fish and crustacea. The nematodes can be cultured by methods described hereinafter to produce amounts sufficient for formulation into suitable compositions for application in aquaculture. Typical compositions for practical use utilise acceptable carrier materials such as foam chips and other solids or semi-solid (flowable) carriers such as gel materials (eg alginate, polyacrylamide, gelatin) materials. Feeding trials have shown that nematodes can support the full development of penaeid larvae at acceptable growth rates and considerably enhance the survival of penaeids during the larval stages of development.

In addition to *P. indicus*, other penaeid species for which the nematodes of this invention may be suitable feeds include *P. monodon*, *P. japonicus*, *P. semisulcatus* and *P. mergulensis*. Nematodes may also be suitable feeds for other crustacean species, such as the coral reef prawns *Lysmata amboinensis*, *L. debelius* and *Stenopus hispidus*, which are extremely valuable to the aquarium industry. Nematodes are also of interest as larval feeds for those commercial fish species which produce very small larvae at first feeding; these include species of rabbit fish, grouper, sea bass and bream. Furthermore, nematodes may be suitable feeds for tropical coral reef fish, such as the Blue Neon Goby, which require very small feed organisms of appropriate nutritional quality.

Example 1 - Culture of Nematodes

Panagrellus redivivus was cultured in 250ml baffled flasks containing 50ml of growth medium comprising 10% (w/w) homogenised kidney, 3.5% (w/w) corn oil and 1% (w/w) yeast extract which had previously been inoculated with the bacterium *Escherichia coli.* An inoculum of 2000 nematodes per ml was added to the flasks which were incubated at 22°C for 10 to 22 days in an orbital incubator. The nematode population reached a maximum of 230,000 nematodes per ml at which point the nematodes were harvested by centrifugation and cleaned to remove any residual medium components by a repetitive process of suspension in clean water, sedimentation under gravity and decantation.

Other suitable growth media for mass culturing of *P. redivivus* can be obtained by substituting kidney in the above medium with animal protein, lecithin, tryptic soy broth, fish autolysate, dried egg or chicken liver.

It is also possible to use these media for mass production of *P. redivivus* in fermenters. An inoculum of 2000 nematodes per ml, which had been produced in baffled flasks, was introduced into a 10 litre fermenter and incubated at 22°C. The population of nematodes reached a maximum of 174000 nematodes per ml between 18 and 25 days, at which point the nematodes were harvested.

Example 2 - Enrichment of the HUFA content of nematodes

P. redivivus and *C. elegans* were each cultured separately in shake flasks on the kidney/corn oil/yeast extract growth medium as described in Example 1. The effect of partially or wholly substituting the corn oil fraction of the growth medium with a fish oil, Capelin oil, was

investigated. The fish oil was used at a proportion of 0, 25, 50, 75 or 100% of the total oil component of the growth medium. The results are summarised in Figure 1 and Table 1. Both nematodes can grow well on all levels of fish oil but survival of the nematodes was significantly better on media containing 25 to 50% of the oil component as fish oil. The HUFA content, and particularly that of the desirable docosahexenoic acid (22:6 ω3) and eicosapentaenoic acid (20:5 ω3), of nematodes increased with the increasing fish oil content of the growth medium. Thus it is possible to increase the HUFA content of nematodes by a mass culturing method which is capable of producing suitable quantities of nematodes for commercial applications. Nematodes with an increased content of HUFA are herein described as enriched nematodes (EN).

Table 1. HUFA Content of *P. redivivus* and *C. elegans* grown on media containing capelin oil.

Proportion of Capelin Oil (% of total oil content)	HUFA Content (% of total lipid)			
	22:6 ω3		20:5 ω3	
	P. redividus	*C. elegans*	*P. redividus*	*C. elegans*
0	0.09	0.18	1.97	2.20
25	0.39	1.90	5.74	3.20
50	0.36	1.73	6.82	5.72
75	0.49	-	7.08	-
100	0.51	2.06	7.40	7.05

Capelin oil can be replaced with other fish oils such as cod liver oil, mackerel oil, salmon oil, dog fish oil, sardine oil, herring oil, and Marila™ (a liquid natural self-emulsifying concentrate containing marine lipids and carotenoids). Good growth of P. redividus is obtained in media where corn oil is replaced as the source of triglycerides wholly or in part by any of the

aforementioned fish oils, except Marila™ which can only be used as up to a 50 % substitution for corn oil. Use of these oils results in nematodes with enhanced HUFA content (Table 2). Dog fish oil is a particularly good source of triglycerides, and when used as a 50% substitution for corn oil gives nematode yields significantly greater than that obtained with corn oil as the sole source of triglycerides (Table 3).

Table 2. HUFA Content of *P. redividus* grown on media containing fish oil and/or corn oil.

Oil	HUFA Content (% of total lipid)	
	22:6 ω3	20:5 ω3
100% Corn	0.80	1.29
50% corn + 50% Capelin	4.00	5.24
50% corn + 50% Marila™	4.39	6.00
50% corn + 50% Cod liver	2.79	5.33

Table 3. Yield of *P. redivivus* grown on media containing dog fish oil

Proportion of dog fish oil (% of total oil content)	Yield* (Nematodes per ml)
0	108,000 - 134,015
25	159,750 - 189,000
50	181,000 - 212,000
75	92,000 - 128,250
100	122,000 - 155,000

* Measured at 5-8 days incubation

Example 3 - Pigmentation of nematodes

P. redivivus was grown as described in Example 1 on a kidney/corn oil/yeast extract medium to which the pigment carophyll pink had been added at a concentration of 0.5 1.0, 1.5 or 2.0%

(w/w). Carophyll pink is a product of Hoffman La Roche and contains 8% (w/w) of the pigment astaxanthin encapsu[redacted] in gelatin capsules. When nematod[redacted]evels had reached approximately 150000 nematodes per ml, they were harvested, washed, homogenised in acetone and the concentration of astaxanthin in the nematodes measured spectrophotometrically. No effect of carophyll pink on nematode growth was observed at concentrations of 2% (w/w) or below. The results of pigmentation of nematodes are summarised in Table 4. A concentration of 1.5% (w/w) carophyll pink in the medium gives the optimum astaxanthin concentration in the nematodes.

Table 4. Astaxanthin incorporation into *P. redivivus*

Astaxanthin concentration in growth medium (% w/w)	Astaxanthin concentration in nematodes (µg/g dry wt.)
0.5	1.05
1.0	1.37
1.5	1.43
2.0	1.38

Example 4 - Pigmentation and enrichment of the HUFA content of nematodes

P. redivivus was grown as described in Example 2 on a medium containing capelin oil as 25, 50, 75 or 100% of the total oil component, and to which the pigment carophyll pink had been added at a concentration of 0.5, 1.0, 1.5 or 2.0% (w/w). When grown on a medium containing 50% of the total oil component as capelin oil and 1.5% carophyll pink, at harvest the nematodes contained 1.47 µg astaxanthin per gram dry weight and 9.17% and 0.41% of total lipid as eicosapentaenioc acid (20:5 ω3) and docosahexenoic acid (22:6 ω3) respectively. Nematodes grown by this method are described herein as pigmented enriched nematodes (PEN). Nematodes grown on 100% corn oil in the absence of carophyll pink contained no detectable levels of astaxanthin or docosahexenoic acid and only 1.25% of total lipid as eicosapentaenioc acid.

Example 5 - Formulation of nematodes

P. redivivus nematodes, which had been grown as described in Example 2 or 4, were harvested by centrifugation and washed in water by a repetitive process of settling and resuspension in fresh water until the nematodes were free of residual growth medium. The washed nematodes were concentrated by centrifugation to produce a nematode aqueous paste which contained in the range of 0.1×10^6 to 2.0×10^6 nematodes per gram of paste.

The paste was packed into high density polyethylene bags containing polyether polyurethane foam chips to give a nematode concentration in the range of 2.0×10^6 to 20.0×10^6 nematodes per gram of foam chips. Water was added to the bags in the range of 1 to 20 ml per gram of foam chips.

Nematodes were also encapsulated in alginate beads by mixing the nematode paste with a chilled 2% (w/v) solution of sodium alginate in water. The mixture was added dropwise to a chilled 1.47% (w/v) solution of $CaCl_2$ and beads left to form. The beads formed were approximately 3mm in diameter and each contained at least 2000 nematodes.

Example 6 - Penaeid feeding trials with HUFA-enriched nematodes (EN)

Feeding trials with *Penaeus indicus* larvae were performed in 2 litre round-bottom glass flasks incubated in a water bath at 28°C. Sea water salinity was adjusted to 25-26ppt and filtered to 0.2µm prior to UV irradiation. Aeration was sufficient to maintain the penaeid larvae in constant but gentle motion. 10 larvae were measured and staged from each flask on a daily basis. 50 and 100% water exchanges were performed on alternate days and total survival was measured every 48h. Larvae were stocked at the Z1 stage of development at 100 larvae per litre.

P. indicus larvae were fed at 30 nematodes per ml during the Z1 to Z3 stages of penaeid larval development and then at 60 nematodes per ml during the M1 to PL1 stages of development. A comparison was made between non-enriched nematodes (NEN), grown on a corn oil medium as described in Example 1, and enriched nematodes, grown on corn oil/fish oil media as described in Example 2. The media contained equal proportions of corn oil and one of the following fish oils: cod liver (CLO), Marila™ (MAR) and Capelin (CAP). The nematodes were formulated in foam chips as described in Example 5. Nematodes were extracted from the bags

prior to use by placing foam chips in a beaker of water, squeezing the nematodes and thoroughly rinsing the sponges. The control feed consisted of 60 algal cells per µl (a mixture of *Tetraselmis chuii* and *Skeletonema costatum*) from the Z1 to M2 stages of development followed by 5 *Artemia* per ml from the M1 to PL1 development stages.

The results for penaeid growth and survival are presented in Figures 2 and 3 respectively. When the nematodes enriched with different lipid sources were fed to *P. indicus* larvae, the lowest mortality rate was observed among the larvae fed the CLO nematodes (2.3% day/l) during protozoeal stages. Larvae fed MAR and EN (Capelin oil) nematodes also displayed significantly lower mortality rates (3.97-5.40 % day/l) compared to those fed NEN nematodes (7.55% day/l) and control live mixed algae (6.56% day/l). No significant difference ($P>0.05$) in the mortality rate was found between the larvae fed either HUFA-enriched or non-enriched nematodes during mysis stages. However, larvae receiving the HUFA-enriched nematodes resulted in significantly higher survivals (69-77%) than those on NEN nematodes (53.5%) and the control diet (54.83%) at metamorphosis to the first PL stage. Larvae fed on EN nematodes exhibited equal growth rates (0.574 mm day/l) to those fed live mixed algae (0.576 mm day/l) between PZ1 and PZ3/M1 stages. Highest larval growth rate between M1 and PL1 was, however, achieved with MAR nematodes (0.425 mm day/l). It appears that CLO was a suitable lipid source to improve survival whereas Capelin oil and Marila oil favour the growth of *P. indicus* larvae. Larvae fed HUFA-enriched nematodes developed into M1 and PL1 stages one day earlier than those fed non-enriched nematodes.

These results demonstrate quite clearly that HUFA-enrichment of nematodes enhances both survival and growth of the *P. indicus* larvae.

Example 7 - Penaeid feeding trials with HUFA-enriched and pigmented nematodes (PEN)

Feeding trials with *Penaeus indicus* larvae were performed as described in Example 6. HUFA-enriched and pigmented nematodes were produced as described in Example 4 in a growth medium containing equal proportions of corn oil and cod liver oil as the source of triglycerides. The results are described in Figures 4 and 5.

In trials with pigmented nematodes the improved pigmentation of the larvae was apparent after just 24 hours of feeding. During the zoeal stages pigmented and HUFA-enriched nematodes

(PEN) resulted in superior pigmentation and promoted the lowest mortality (2.83%/day) in comparison to placebo pigment/HUFA-enriched nematodes (PLC) which had a mortality rate of 6.30%/day. In comparison, HUFA-enriched nematodes (CLO) had a mortality rate of 4.22%/day with the algae/*Artemia* controls demonstrating the highest mortality rate of 17.68%/day. The PEN diet resulted in 88% survival to metamorphosis while PLC, CLO and the control diets gave final survivals of 78, 79 and 9% respectively.

Larvae fed PEN displayed superior growth only during the Z1 to Z3/M1 stages. However, total lengths of PEN fed larvae at metamorphosis were not significantly different from those receiving the non-pigmented nematodes (EN). Thus PEN diet promoted significantly better growth rates (0.455 mm/day) than either PLC or CLO nematode feeds (0.415-0.440 mm/day) during the zoeal growth phase but this pattern was not maintained during the mysis stages.

CLAIMS

1. Nematodes grown on a liquid culture medium containing a fish oil.

2. Nematodes grown on a liquid culture medium containing a fish oil and a pigment, for example a pink pigment, acceptable to marine organisms amenable to aquaculture.

3. Nematodes according to claim 1 or 2, of the genera *Panagrellus, Turbatrix, Caenorhabditis, Monohystera, Theristis, Rhabditis, Phasmarhabditis, Heterorhabditis, Steinernema, Chromadora, Enoplus, Communis* and *Metoncholaimus.*

4. Nematodes according to claim 1 or 2, of the species *Panagrellus redivivus.*

5. An aquaculture feed composition comprising nematodes according to any of Claims 1 to 4 and a suitable carrier material.

6. A process of culturing nematodes in which the culture medium is a liquid containing a fish oil.

7. A process according to Claim 6, in which the oil component of the medium comprises a fish oil and a vegetable oil, such as corn oil.

8. A process according to claim 6 or 7, in which the fish oil constitutes from 25 to 50% by weight of the oil component of the medium.

9. A process for aquaculture of marine organisms, in which an aquaculture feed composition is used which comprises nematodes grown on a liquid culture medium containing a fish oil.

10. A process according to claim 9, in which the marine organisms are penaeid species.

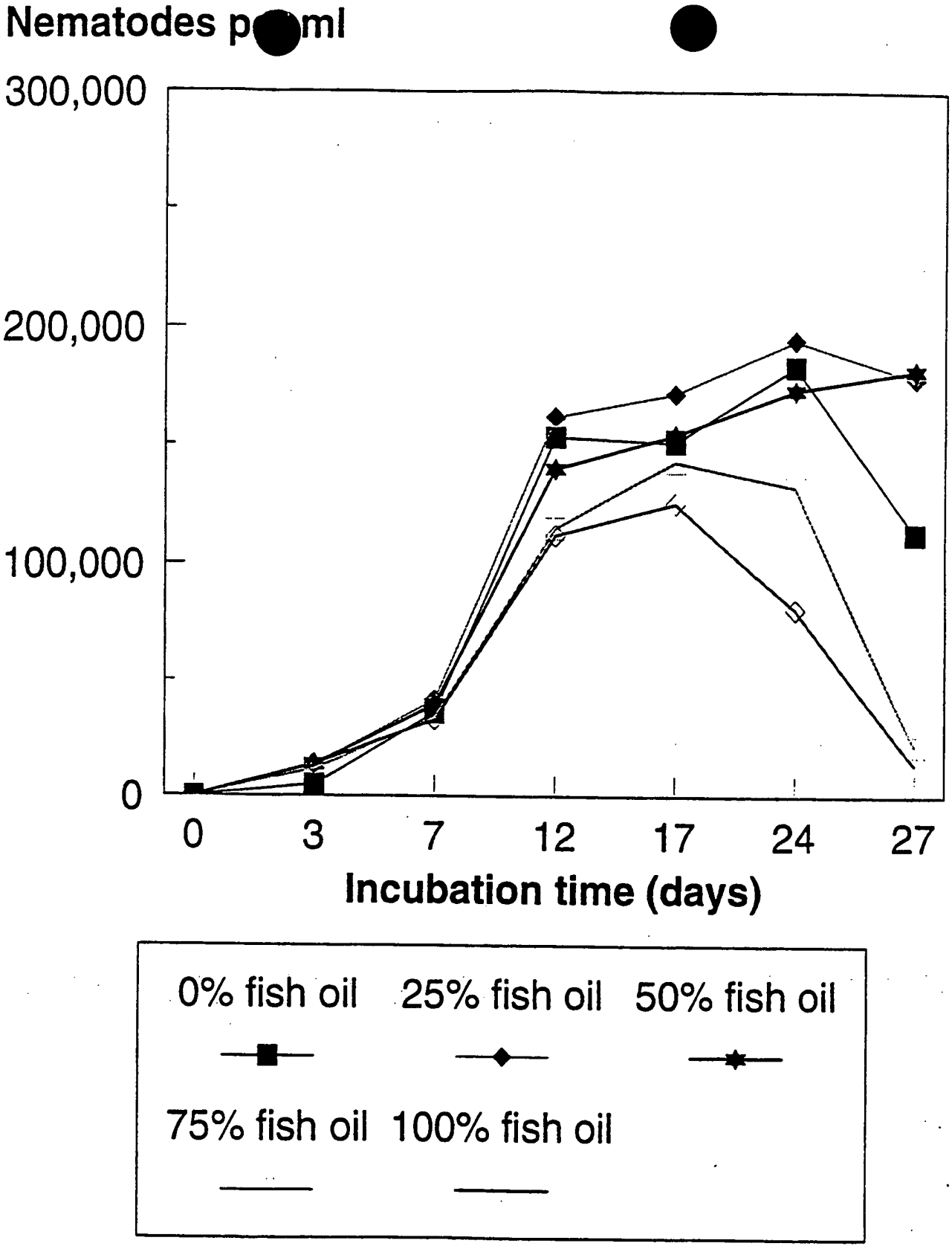

Figure 1. The growth of Panagrellus redivivus on different levels of fish oil.

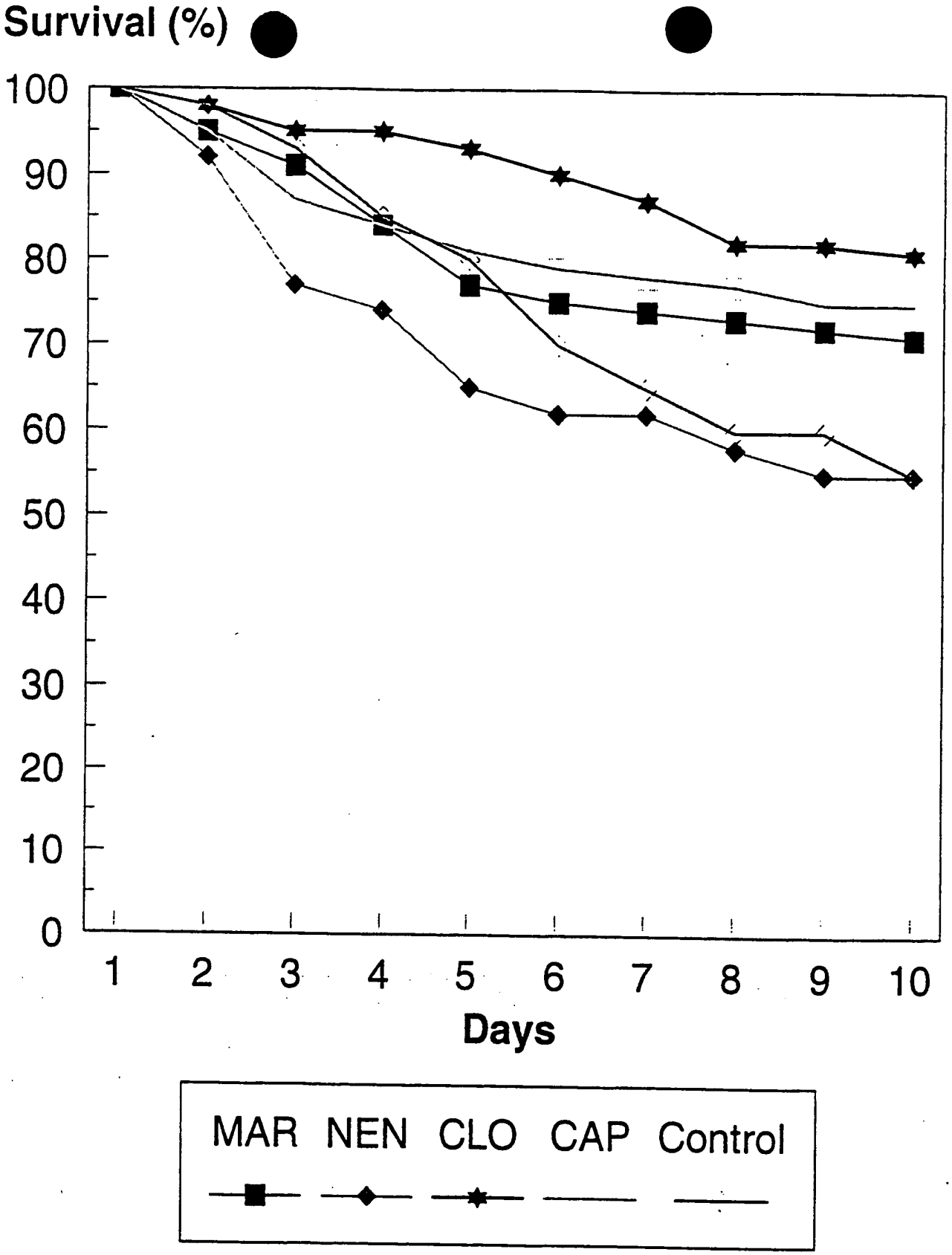

Figure 2. Survival of P. indicus larvae fed non-enriched, HUFA-enriched nematodes or a control diet.

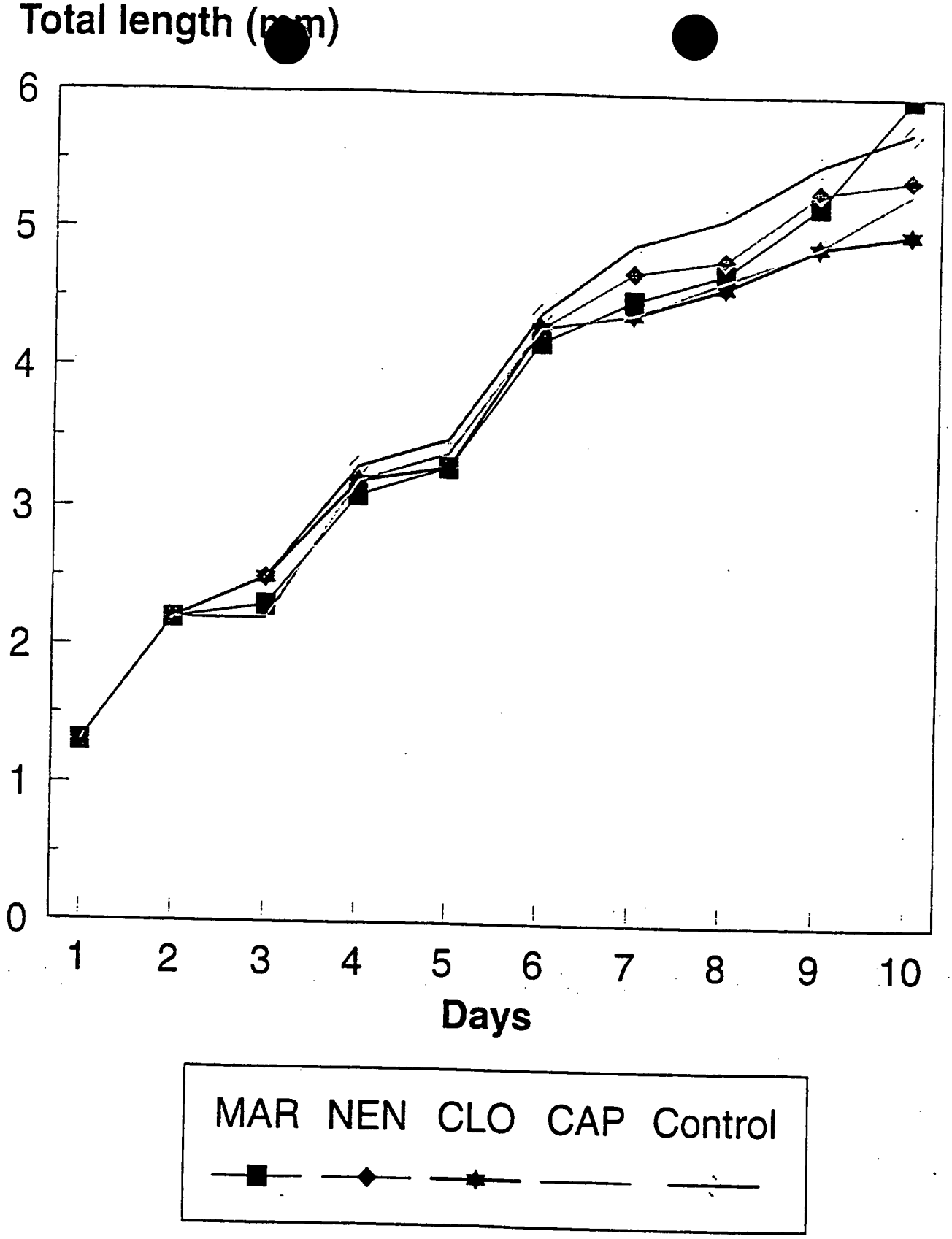

Figure 3. Growth of P. indicus larvae fed non-enriched, HUFA-enriched nematodes or a control diet.

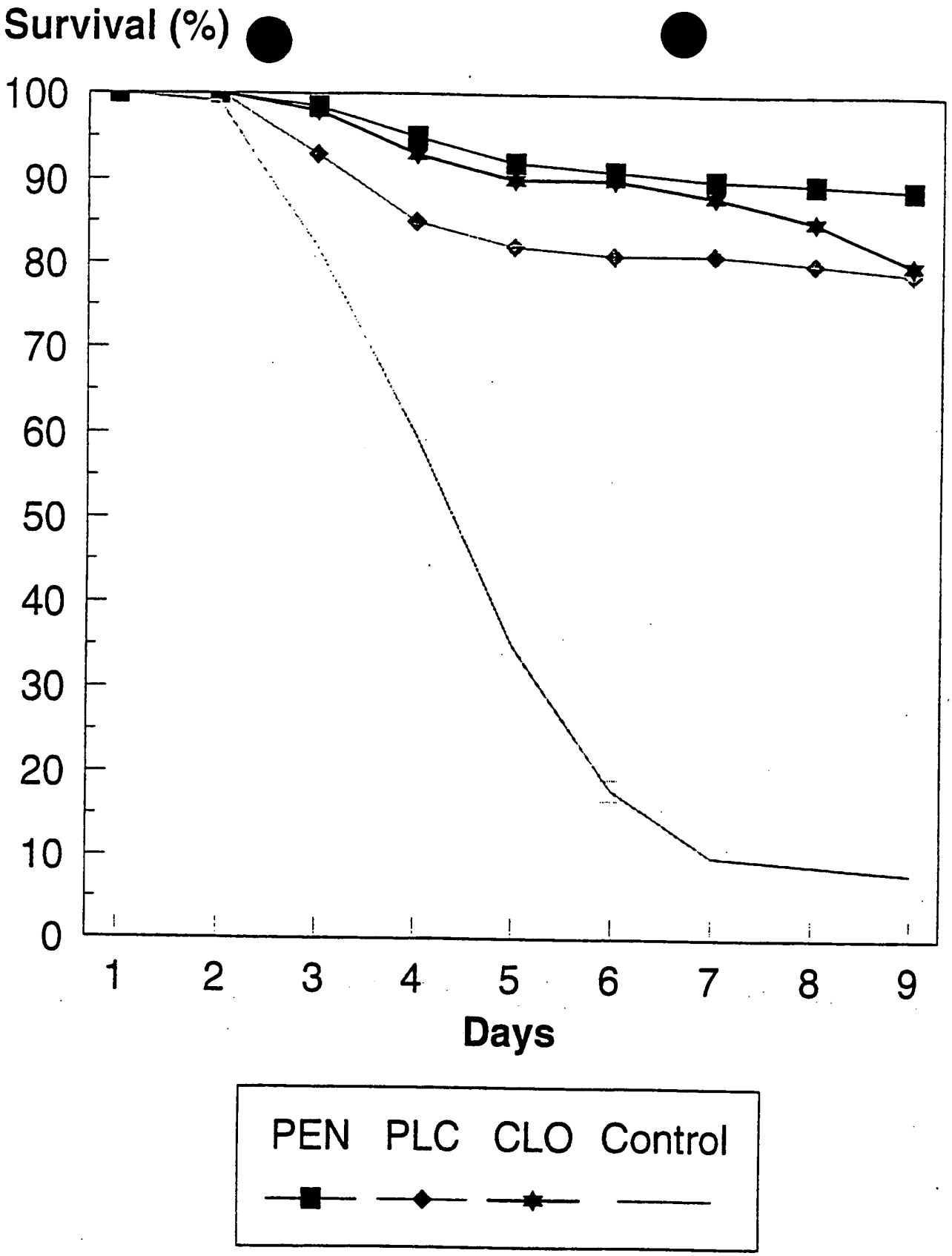

Figure 4. Survival of P. indicus larvae fed pigmented, enriched nematodes (PEN), placebo pigment, enriched nematodes (PLC), cod liver oil enriched nematodes (CLO) or a control live diet.

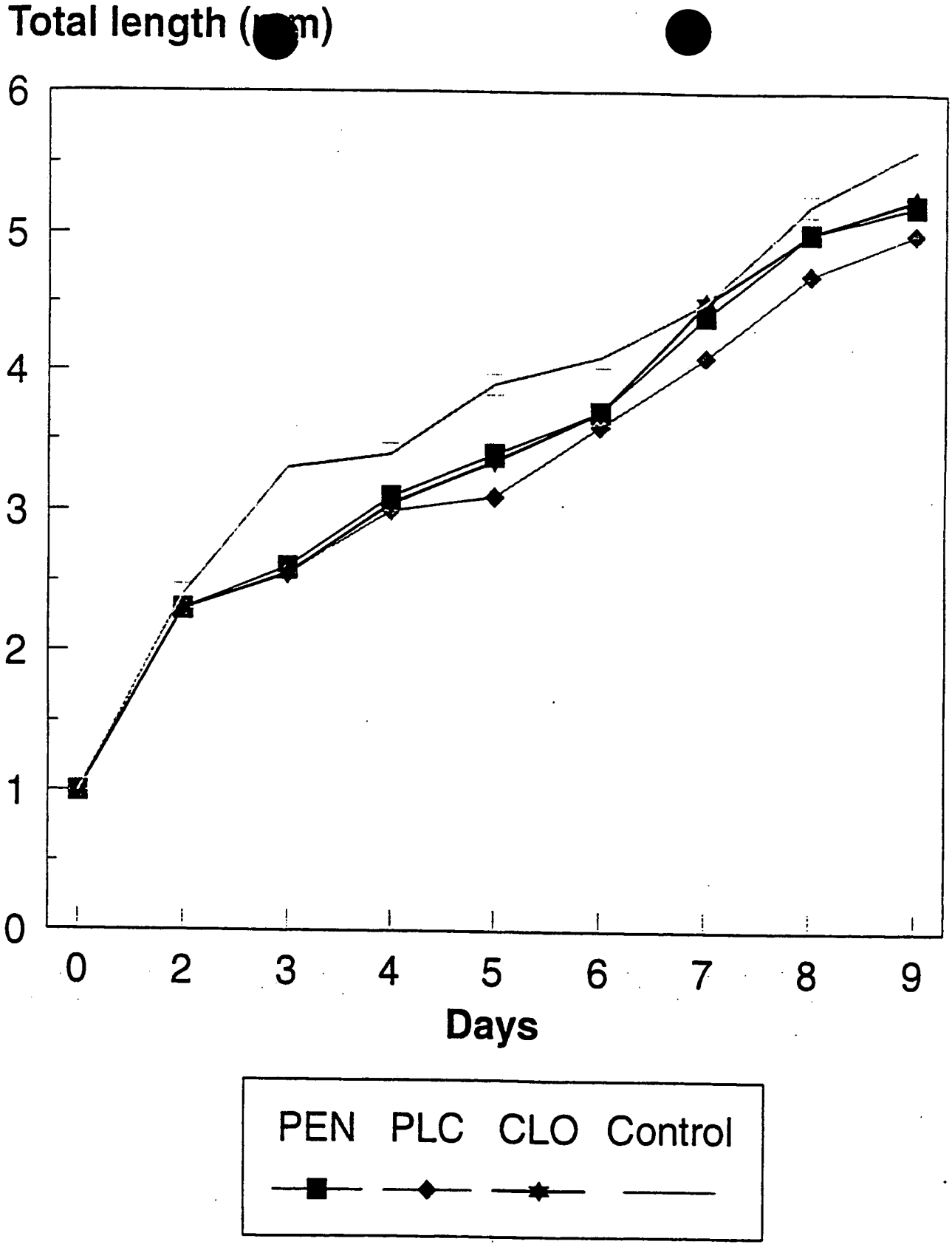

Figure 5. Growth of P. indicus larvae fed pigmented, enriched nematodes (PEN), placebo pigment, enriched nematodes (PLC), cod liver oil enriched nematodes (CLO) or a control live diet.

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/GB 95/00021

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01K67/033 [redacted] 23K1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE WPI Week 9444, Derwent Publications Ltd., London, GB; AN 94-353612 & JP,A,6 276 892 (OJI PAPER CO) 4 October 1994 see abstract --- -/--	1,3,6

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Date of the actual completion of the international search	Date of mailing of the international search report
20 February 1995	07.03.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Dekeirel, M

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PCT/GB 95/00021

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF THE WORLD AQUATIC SOCIETY, vol.23, no.1, 1992 pages 89 - 95 DAVID B. ROUSE ET AL. 'Enhancement of the Fatty Acid Composition of the Nematode Panagrellus redivivus Using Three Different Media' cited in the application see page 89, Abstract see page 90, column 1, paragraph 2 see page 91, column 2, paragraph 3 - page 94, column 1, paragraph 1	1,3,6
A		4,5,9,10

Y	WO,A,89 04602 (BIOSYS) 1 June 1989 cited in the application see page 9, line 33 - page 10, line 5 see claim 1	1,3,6
A		7,8

A	WO,A,93 00816 (AGRICULTURAL GENETICS COMPANY LIMITED) 21 January 1993 see examples 4,5	1,3,5

A	CAHIERS DE BIOLOGIE MARINE, vol.16, no.5, 1975 pages 685 - 693 JOHN H. TIETJEN ET AL. 'Axenic culture and uptake of dissolved organic substances by the marine nematode, Rhabditis marina Bastian' see page 688; table 1	1,3

A	JOURNAL OF THE WORLD AQUACULTURE SOCIETY, vol.20, no.2, 1989 pages 61 - 71 JAMES M. BIEDENBACH ET AL. 'Use of the Nematode Panagrellus redivivus as an Artemia Replacement in a Larval Penaeid Diet' cited in the application see page 62, column 1, paragraph 2	5,9,10

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